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EXTRACTION AND CHARACTERIZATION OF ALGOIL FROM SELECTED MARINE MICROALGE AND THE ROLE OF ASSOCIATED BACTERIA ON FATTY ACID PROFILE

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ABSTRACT

This work was conducted to study the role of selected associated bacteria on the physic-chemical nature of algal oil and PUFA. The two associated bacteria based on the performance in encouraging the growth of microalgae were selected and allowed to co-exist with the culture of micro algae such as *Skeletonema costatum* and *Isochrysis galbana*. The cells were harvested by flocculation and the oil was extracted. The effect of different phases of growth on oil yield, physical and chemical characters of extracted oil, total oil yield and the type of PUFA with and without associated bacteria were studied. Among the six species of bacteria isolated from the log phase culture, *Pseudomonas* sp. and *Micrococcus* sp. encouraged the growth of micro algae. The total oil yield and total carotenoid production were found to be increased by the co-culture of associated bacteria. The associated bacteria increased the quantity of vitamin –A and -E and there was no effect observed in the mineral content of micro algal oil with the co-growth of selected bacteria; but proved its positive role in PUFA increment of micro algae studied than the control without co-existence of selected bacteria. From this study, the positive role of associated bacteria on growth of algae, total oil yield and PUFA increment have been documented and it has opened a new avenue of research in changing the PUFA profile by manipulating the associated bacteria.

KEYWORDS: Algoil, Associated Bacteria, Isochrysis Galbana, Microalgae, PUFA, Skletonema Costatum

INTRODUCTION

Microalgae are the efficient biological factories capable of taking carbon from waste and converting it into a high density liquid form of oil (natural oil). Microalgae contain significant qualitative form of lipids (fats and oil) with compositions similar to those of vegetable oils (Casimir C.Akoh and David B, 2002). The lipids of algal species are also rich in fatty acids, such as C^{16} and C^{17} and C^{18} derivatives. While it is common to find levels of 20 - 50% lipids on a dry basis, on occasion the quantities of lipids found in microalgae can be extra ordinarily high (Sheehan,J et.al.1998). Concentration of hydrocarbons in the dry matter may exceed 50% under certain conditions, mean while, the levels of 15 - 35% lipids on a dry basis were found in vegetable oils (Metting, F.B,1996 and Spolare, P et al.,2006).

Microalgae are a promising source of PUFA due to its superior lipid stability and naturally rich in antioxidants, carotenoids and vitamins. The high nutritional value and its ability to synthesize and accumulate PUFA only proves that microalgae play an important role in aquaculture (Patil et al., 2006 and Gouveia et al., 2008) The competition between microbes for resources to be converted in to energy and cellular components essential for bacterial growth is a crucial factor limiting and affecting the composition of algal associated bacterial communities (Zaady,et al.,2000). Moreover, the structure of such communities may change successfully as a function of substrate utilization, which depends on the physiological status of the micro algae (Levich A.P.,1996).Additionally, those physiological features may directly influence upon the quality and quantity of micro plant exudates that can be utilized by the bacteria. There by some changes in the fatty acid profiles can be observed directly resulting from shifts in the microbial community structure (Levich A.P., 1996).

Bell et al., (1974) demonstrated that Pseudomonad grows well to a high steady state with co-culture of *Skeletonema costatum* both in batch and continuous culture. While in the absence of alga viable bacterial count was significantly lower. Thus, suggest that Pseudomonad growth is most like supported by the extracellular organic compound and dead algal cell. It has been stated that the role of extracellular organic compound (EOC) secreted by algae sustained the creation and maintenance of a phycosphere effect, showing that microbial utilization of these compound can result in stimulation of physiologically specific bacterial types, likewise bacterial mineralization developed phytoplankton population (Bell et al. 1974).

Interactions between algae and their associated bacteria, either in the plankton or in biofilms, may alter algal sensitivity to contaminants. The associated bacteria of microalgae reduces the copper toxicity in the copper contaminated water and help the algae to get tolerance for copper contamination However, the presence of bacteria had either no pronounced effect on or led to a reduction of the specific death rates of *D. brightwellu*. Rematerialized ammonium was probably partly utilized by N-starved cells of *D. bnghtwellii*, leading to reduced death rates of the algal cells. Bacteria thus indirectly prolonged survival of *D. bnghtwellii* population under N starvation. This study shows that bacteria can affect phytoplankton survival, nutritional composition and reserved oil which in turn may influence algal species succession (Levy JL et al.2009 and Corina P. D. et al .1998)

Algae acquire Vitamin B_{12} through symbiotic bacteria and these bacteria can change the chemical constitution of the micro algae even the lipid composition of its cell (Croft, M., T. et al.2005 and.Robert,Z. 2002) also found the changes in fatty acid profiling of micro algae based on microbial association. There is increasing evidence that there are specific bacterial taxa associated with phytoplankton (Green, D.H.,L.E. Liewellyn, 2004 and Jasti.S.M,E.Sieracki 2005) suggesting the presence of specific selective mechanism and implying that the bacteria have some function to the benefit of the algae. The heterotrophic bacteria can offer CO_2 which micro algae require for photosynthesis and at the same time, they can obtain organic materials and O_2 produced by micro algae (Zhou.w, et al.,2009).

Bacteria living on the surfaces of marine micro-algae or “phycosphere” produced secondary metabolites, which can inhibit the growth of other competitive bacteria. In this case, highly competitive environment where space and availability of nutrients are limited might be a selective force, which may lead to the evolution of a variety of effective adaptation in several algal-associated bacteria (Boydet al., 1999). Additionally, many researchers have suggested that positive effects of bacteria on culture microalgae have been documented (Munro et al., 1995; RicoMora and Voltolina,1998; Hirayama,1996). Unique symbioses that develop from the combination of a specific micro-algae and bacteria have been documented. Nevertheless, successful establishment of symbiotic bacteria remains enigmatic. To date, little is known about the factors controlling algae and bacteria interactions in aquatic ecosystem.

Recently, it has been reported that association of bacteria and algae might be due to spatial, temporal (Grossart et al., 2006) and organic matter produced by different types of algae, which cause shifts in bacterial species composition. The main purpose of this study was to understand the role of associated bacteria on improving PUFA and to know the effect of these bacteria on physico chemical nature of the algal oil.

MATERIALS AND METHODS

Collection of Marine Microalgae

Two marine microalgae such as *Skletonema costatum* (Cleve) and *Isochrysis galbana* (Parke) were purchased from Central Marine Research Institute (CMFRI), Tuticorin, Tamil Nadu, India. The collected algal samples were then brought to the laboratory for stock culture and mass culture studies.

Stock Culture Maintenance of Microalgae

The collected algal cells were multiplied in 5 ml test tube and then transferred to the test tubes and conical flasks. The culture media used in the stock culture was Walne's medium (Walne. P.R, 1970). The stock culture was provided with 2000 lux fluorescent light and no aeration.

Culture Media

The enriched media used in the present study was Walne's Medium

Tank Preparation for Mass Culture of Marine Microalgae

The FRP tanks of two hundred and fifty liters capacity were used for mass culturing of algae. The tanks were rinsed with soap water and washed thoroughly with the tap water.

The sea water enriched with Walne's medium was filled in the tank. Then, 10-20% of the inoculum of growing phase was added in to the respective mass culture tanks. Finally the culture tanks were placed at the direct sunlight with continuous aeration. The growth rates of the algae were measured in every 6 hour interval by taking sample from the mass culture tank and counted the cells by using improved Neubauer chamber (haemocytometer).

Isolation and Screening of Associated Bacteria

The bacteria growing in the algal culture were isolated from the fast growing microalgae of mid log phase by serial dilution and plated on bacterial culture medias such as Zobal Marine Agar, Tri Citrate Bile Salt Agar (TCBS) and Pseudomonas Agar (Himedia, India). The isolated bacteria were allowed to coexist during the growth of the algae. The bacteria that offered the maximum growth for algae during the co-existence were selected to study the role of these bacteria on HUFA yield and other physic chemical characters of oil

Identification and Selection of Bacteria

The isolated bacteria were identified by standard biochemical test as well as with the aid of Bergey's Manual of systematic bacteriology (Robert et al., 1957)

In order to study the role of algal associated bacteria for the yield of PUFA and other fatty acids, the *Pseudomonas* sp. and *Micrococcus* sp. were selected because they encouraged the growth of the algae in the mid log phase. The concentration of cells was made as 1 lakh cells/mL in the sterilized seawater by dilution method and hence the desire concentration of bacterial cell suspension was prepared.

From the cell suspension, 1 mL of bacterial inoculum was pipetted out and inoculated in to the micro algal culture of lag phase. The algal culture was maintained at 30 ppt salinity, 30 ± 2^{o}C temperature and 2000 lux light intensity. The nutrient supplied was Conway medium. In the same manner, another batch of algae grown without any bacteria by UV irradiation continuously for 15 minutes before the experiment was also studied as control. The algal cells from both the bacteria inoculated and UV irradiated were harvested at late log phase and subjected for further studies.

Preparation of Axenic Algal Culture

The axenic algal culture was prepared by UV irradiating the algal culture taken in conical flask and exposed to UV light for 5, 10,15,20,25 minutes. The culture after the respective exposure was allowed for bacterial viable count by sub culture. Among the different time of UV exposures, one did not show any bacterial colony on sub culture and not affected the growth of algal cells was selected as the suitable UV exposure time needed for making axenic algal culture

Harvest of Algal Biomass

Determination of Aluminium Sulphate Concentration Required to Flocculate the Algal Cells

In order to calculate the quantity of aluminium sulphate needed to flocculate the algal culture, 1 litre culture at late log phase were taken in 10 beakers of 2 litres capacity. Various concentrations of powdered Aluminium sulphate (0.01, 0.02, 0.03, 0.04 and 0.05 $g.L^{-1}$ was added into the aliquots of 10 beakers. The culture taken in the beakers were thoroughly stirred to get through mixing of Aluminium sulphate. All the beakers were kept undisturbed for 10 minutes. In a particular concentration of alum treatment where >90% of the cells settled was considered as the optimum concentration for aluminium treatment. In this way, the quantity of Aluminium sulphate required to flocculate the entire volume of the algal culture was determined. As the pH change is the main mechanism for flocculation, the pH of the medium was measured before and after the application of alum.

Application of Alum

Based on the volume of culture in a particular tank, the quantity of alum requirement was determined. The required quantity was taken and dissolved in water. This solution was sprayed on the outdoor algal culture tank during heavy aeration. The heavy aeration was performed continuously for 30 minutes. After 30 minutes, the aeration was cut off and left the culture undisturbed for another 1 h. After the flocculation was observed, the water layered above the flocculated mass was siphoned out.

The harvested cells were repeatedly washed with fresh water to remove the Aluminum sulphate. The pH was measured to ensure that there is no alum residue in the algal powder. The algal mass after complete removal of alum were collected individually and spread in white metal trays and allowed for drying in a oven at $45^{o}C$. After complete drying, the algal biomass was scraped out and taken for further experiments.

Extraction of Algal Oil from Microalgae

The algal oil (lipids) were extracted by a modified method of Folch et al,described by Bligh and Dyer. The extraction solvents contain 0.1% (w/v) of butylated hydroxyl toluene (BHT) in order to avoid oxidation. Hundred grams of algal powder was taken in a 2 litre beaker. To this 100 mL of distilled water was added and mixed. Two hundred mL of chloroform and four hundred mL of methanol (1:2) was added and the mixture was homogenized for 2 min while being cooled in ice. Then 200 mL of chloroform was added again and homogenized for one minute, followed by the addition of 200 ml of distilled water, and finally homogenized for 30 seconds.

The mixture was centrifuged at 2000 rpm for 20 min. The aqueous layer was removed by suction. The chloroform fraction was evaporated using rotary evaporator to remove the chloroform completely. The Aluminium sulphate residues present in the oil was removed by repeated mixing with hot water and centrifugation. Oil obtained in this process was kept in screw capped clean sterile glass bottles.

Solidification Point (Immanuel.G, et al, 2002)

A test tube containing ten grams of solid algoil was kept in a water bath for complete melting. Then the test tubes with oil were again cooled down for further solidification and at the point of solidification, the temperature was noted using thermometer.

Specific Gravity of Algoi (Immanuel.G, et al, 2002)

The specific gravity was measured using the method described by Immanuel et al., 2002.

Determination of Acid Value and Free Fatty Acid

The acid value was determined by titrating directly the oil in alcoholic medium against standard potassium hydroxide / sodium hydroxide solution Immanuel.G et al., (2002)

Analysis of Carotenoid (Carreto,J.I, J. A. Catoggio (1977)

The extracted algoils were taken for the analysis of carotenoids. The algoil samples were dissolved in known volume of hexane. These samples were scanned under the visible range of UV-visible spectrophotometers (Techcomp 8500, Taiwan). From this, total carotenoid content as well as β-carotene were estimated using the following formula.

$$\text{Total carotenoid} = \frac{\text{Maximum Absorbance } 456}{259.2 \times \text{sample weight}} \times \text{volume of sample} \times 100$$

$$\beta\text{-carotene (\%)} = \frac{\text{Absorbance } 456}{196 \times \text{sample weight}} \times \text{volume of sample} \times 1.25 \times 100 \times 0.84$$

Analysis of Vitamins (Katharine H et al.1935)

The fat soluble vitamins such as A, D, E, K in the extracted algoil samples were determined by standard procedure described by *Katharine H et al.1935.*

Mineral Content (R.P. King, 1979)

The minerals such as copper, iron, sodium, potassium, magnesium, phosphorus, and zinc contents of the extracted algoil samples were determined by standard procedure described by R.P. King (1979).

Heavy Metals *(*A.-H. Buo-Olayan, M. N. V. Subrahmanyam, 1996)

The heavy metals such as mercury, arsenic, lead and cadmium contents in the extracted algoil samples were determined using standard procedure described by A.-H. Buo-Olayan, M. N. V. Subrahmanyam (1996).

Analysis of Fatty Acid

Gas chromatography coupled with mass spectrometry (Horman.. H,I. Traitler in 1989) was used for the fatty acid quantification. The GC-MS used is the Namag R 10-10C combined gas chromatograph controlled by a digital PDP11-23 plus system (Delsi-Nermag, Argenteuil, France). Two different ionization methods were employed. EI and PCI were using NH_3 (John B. Westmore et al.in 1986) (Air liquid, Le Plessis Robinson, France), as a reagent gas at a pressure of 0.1 Torr in the ion source. Operating conditions were as follows: filament current 200 mH, ionization energy 90 eV, ion source temperature 120ºC.

Quantification of Different Fatty Acids at Different Growth Phase of Algae

This work was done to know the percentage of different fatty acid during different phases of algal growth. The two algal species *Isochrysis* and *Skletonema inoculated with Psedomonas* were cultured and harvested at 12, 72 and 108 h after the inoculation of algae. During this period, the bacterial count was also recorded in both the microalgal species. The cells were dried and extracted the oil and analyzed the oil for percentage of PUFA. The algae developed without bacterial inoculation was treated as control and that was also harvested at three phases of growth and extracted the oil.

Five replicates were provided for the above all experiments. The experiments were also repeated three times for all the parameters to get consistent data. The outdoor control was made axenic by sending the algal culture through bactericidal UV system with the retention time of 5lit/min. and the tanks were covered with transparent polyethylene cover to prevent further contamination with bacteria. The total bacterial count was made every day morning and evening to know any contamination after UV treatment.

Statistical Analysis

The data obtained in the present study were expressed as mean ISD and were analyzed using one way anova (ANOVA) at the 5% significant level. Further a multiple compensation test (Student-Newman-Keuls, SNK test) was conducted to compare the significant differences among the treatment using computer software Statistica 6.0 (statsoft; http://www.statsoft.com).

RESULTS

Associated Bacteria on Algal Growth

The five associated bacteria such as *Halomaonas* sp.,*Pseudomonas* sp.,*Vibrio* sp.,*Pseudoalteromonas* sp., and *Micrococcus sp.* were inoculated in to the UV treated *Skeletonema* and *Isochrysis*., and allowed to grow. When the culture reached the mid log phase the cell count were taken.Among the five bacteria tested, the *Pseudomonas* sp. helped for the maximum growth of *Skeletonema* and *Isochrysis*. (Table -1)

Table 1: Influence of Different Algal Associated Bacteria on the Growth of Microalgae at Mid Log Phase

Sl. No.	Name of Associated Bacteria Inoculated	Cell Count at Mid Log Phase Cells^{-ml}	
		***Dicrateria* sp. (X10^5)**	***Tetraselmis* sp. (X10^5)**
1	Control (UV treated)	1.93 ± 0.42^{a}	1.96 ± 0.39^{a}
2	*Halomonas* sp.	1.68 ± 0.30 ^{a}	1.68 ± 0.30 ^{a}
3	*Pseudomonas* sp.	3.62 ± 0.31^{c}	3.73 ± 0.58^{c}
4	*Vibrio* sp.	2.89 ± 0.72^{d}	2.27 ± 0.45^{d}
5	*Pseudoalternomonas* sp.	3.32 ± 0.27^{e}	2.49 ± 0.50^{e}
6	*Micrococcus* sp.	3.12 ± 0.39^{f}	3.38 ± 0.72^{f}

Each value is the mean ± SD of three replicates within each column means with different superscript letters in both group are statistically significant (t–test<0.05 and subsequent posthoc multiple comparison with SNK test)

The statistical analysis shows that the influence of bacterial inoculation except *Holomonas* sp. significantly ($P<0.05$) increased the growth of two microalgae when compared with the control

Physico-Chemical Characters

In order to study the role of associated bacteria on physiological characters of algal oil such as solidification point, melting point, specific gravity, moisture content, acid value and fatty acid value were studied. In solidification point, there was no difference found between the *Pseudomonas* sp. introduced micro algal culture and the control (without bacteria). But, it varied based on the species. Melting point also had no variation with the introduction of associated bacteria and without bacteria and it varied based on the species of algae from which the oil was taken. Likewise, the specific gravity, moisture content, acid value and fatty acid value possessed a little influence due to the introduction of associated bacteria.

But, the total carotenoid and β-carotene level showed a positive influence due to the introduction of associated bacteria isolated from the micro algae. The *Skeletonema* when grow with *Pseudomonas* sp. yielded a carotenoid percentage of 3.35 and in the control only 2.40% was recorded. In *Isochrysis* also the variation was noticed between the experimental (3.05%) and control (1.97%).

The percentage of β-carototene due to associated bacterial inoculation increased (1.43%) than the control (1.04%) in *Skeletonema* sp. and the same trend was also observed in *Isochrysis* sp. The associated bacteria also proved its positive role in the quantity of oil yield. The *Skeletonema* algae grown with *Pseudomonas* sp. yielded more oil (20.62 ml/100g) than the control (17.64 ml/100g). In *Isochrysis* sp. also the same effect due to the associated bacteria was observed (Table -2).

Table 2: Physico-Chemical Characters of Extracted Algoil with and without Associated Bacteria (*Pseudomonas* sp.)

Characters	*Skeletonema* sp.	Control	*Isochrysis* sp.	Control
Yield of Oil (ml $^{-100g}$)	20.62 ± 3.22 [a]	17.64 ± 1.72 [b]	25.42 ± 2.64 [a]	18.32 ± 2.47 [b]
Solidification Point (^{o}C)	18.049 ± 0.35 [a]	18.52 ± 0.46 [a]	16.43 ± 0.40 [a]	16.40 ± 0.38 [a]
Melting Point (^{o}C)	28.30 ± 0.40 [a]	28.43 ± 0.37 [a]	27.33 ± 0.12 [a]	27.33 ± 0.15 [a]
Specific Gravity (g)	0.913 ± 0.012 [a]	0.913 ± 0.03 [a]	0.896 ± 0.006 [a]	0.896 ± 0.008 [a]
Moisture Content (%)	10.62 ± 0.06 [a]	10.72 ± 0.07 [a]	07.63 ± 0.02 [a]	07.40 ± 0.03 [a]
Acid value (%)	2.83 ± 0.16 [a]	2.84 ± 0.64 [a]	7.34 ± 0.24 [a]	7.41 ± 0.25 [a]
Fatty Acid Value (%)	1.40 ± 0.016 [a]	1.45 ± 0.017 [a]	0.902 ± 0.0016 [a]	0.93 ± 0.004 [a]
β Carotene (%)	1.43 ± 0.02 [a]	1.04 ± 0.03 [b]	1.72 ± 0.01 [a]	1.32 ± 0.05 [b]
Total Carotenoid (%)	3.35 ± 0.02 [a]	2.40 ± 0.053 [b]	3.05 ± 0.132 [a]	1.97 ± 0.45 [b]

Each value is the mean ± SD of three replicates within each column means with different superscript letters in both group are statistically significant (t–test<0.05 and subsequent posthoc multiple comparison with SNK test)

The t test conducted between the experimental and control showed that the oil yield, total carotenoid and β carotene were significantly (P<0.05) increased by the associated bacteria in both the microalgae studied.

Associated Bacteria and Algal Vitamin Content

In order to study the influence of associated bacteria on level of fat soluble vitamins in the algal cells, the Psedomonas was inoculated in to *Skeletonema* and *Isochrysis* culture. The inoculated bacteria increased the vitamin A and E in *Skeletonema* and *Isochrysis* sp., but no effect was found in vitamin D content in both the species. Vitamin K was absent in both these micro algae (Table-3).

Table 3: Vitamin Content of Algoil from Microalgae Associated with *Pseudomonas* Bacterium

Vitamins	*Skeletonema* sp. (IU/100 g)	Control (IU/100 g)	*Isochrysis* sp. (IU/100 g)	Control (IU/100 g)
Vitamin A	35.55 ± 3.90 [a]	30.43 ± 3.75 [b]	25.42 ± 2.40 [a]	19.52 ± 1.62 [b]
Vitamin D	25.63 ± 2.53 [a]	25.40 ± 1.73 [a]	16.85 ± 1.30 [a]	15.91 ± 2.17 [a]
Vitamin E	18.67 ± 1.63 [a]	14.43 ± 1.55 [b]	10.65 ± 1.30 [a]	8.21 ± 1.05 [b]
Vitamin K	Nil	Nil	Nil	Nil

Each value is the mean ± SD of three replicates within each column means with different superscript letters in both group are statistically significant (t–test<0.05 and subsequent posthoc multiple comparison with SNK test)

The t test conducted between the experimental and control showed that the associated bacteria significantly increased (P<0.05) the production of Vitamin A and E except vitamin D.

Associated Bacteria and Algal Mineral Content

Effect of bacterial association on mineral content of algal was studied. The presence of minerals like calcium, sodium, potassium, iron, magnesium, zinc, sulphur and phosphate in the algal oil did not have any change due to the inoculation of *Pseudomonas* sp. And, the level of such minerals in the oil also had no variation due to bacterial inoculation Cobalt was absent in both *Skeletonema* and *Isochrysis* sp (Table 4).

Table 4: Mineral Content of Algoil Obtained from Microalgae Grown with Symbiotic Bacterium *Pseudomonas* sp.

Mineral	*Skeletonema* sp. (mg^{-100g})	Control (mg^{-100g})	*Isochrysis* sp. (mg^{-100g})	Control (mg^{-100g})
Calcium	132.67 ± 15.21 [a]	130.62 ± 16.42 [a]	127.5 ± 11.60 [a]	126.62 ± 13.67 [a]
Sodium	234.78 ± 12.30 [a]	236.73 ± 14.23 [a]	136.85 ± 15.13 [a]	134.41 ± 16.35 [a]
Potassium	67.64 ± 3.76 [a]	66.28 ± 4.72 [a]	68.52 ± 5.70 [a]	69.05 ± 7.53 [a]
Cobalt	Nil	Nil	Nil	Nil
Iron	0.60 ± 0.162 [a]	0.67 ± 0.14 [b]	0.60 ± 0.13 [a]	0.63 ± 0.12 [a]
Magnesium	0.56 ± 0.094 [a]	0.65 ± 0.073 [a]	Nil	Nil
Zinc	6.44 ± 1.20 [a]	6.20 ± 1.04 [a]	Nil	Nil
Sulphur	3.45 ± 0.76 [a]	3.33 ± 0.50 [a]	3.19 ± 0.072 [a]	3.97 ± 0.06 [a]
Phosphorus	0.96 ± 0.12 [a]	0.89 ± 0.16 [a]	0.26 ± 0.58 [a]	Nil

Each value is the mean ± SD of three replicates within each column means with different superscript letters in both group are statistically significant (t–test<0.05 and subsequent posthoc multiple comparison with SNK test)

The experimental value obtained due to the associated bacteria are non significant (P>0.05) in both the species of micro algae when compared with its control

Associated Bacteria on PUFA Yield in Phase of Growth

The role of associated bacteria on changing the PUFA production was studied by harvesting the algae grown with *Pseudomonas* sp. during its different growth phases like lag phase, late lag phase, log phase, late log phase, stationary phase and late stationary phase. There was an increase in the PUFA level observed in *Skeletonema* co-cultured with *Pseudomonas* than the control (without bacteria). In *Skeletonema*, the maximum PUFA was noticed in late log phase (3.12 g/100g dry weight) and only 1.83 g/100g was found in the control during the late lag phase.

The minimum PUFA levels in experimental and control was 1.72 and 1.07 g /100g respectively, which were found in the lag phase. The *Isochrysis* sp. also had the maximum PUFA in late log phase (1.62 g/100g) where as the control (without *Pseudomonas* co-culture) had 0.96 g/100g and from this experiment it was evident that the associated bacteria positively played a role in the PUFA content in the cells of micro algae. (Table -5)

Table 5: Total PUFA Content of Two Species of Microalgae during Different Phases of Growth When Inoculated with *Pseudomonas* sp.

Phases of Growth	*Skeletonema* sp.	Control	*Isochrysis* sp.	Control
	Quantiity of PUFA*	Quantiity of PUFA*	Quantity of PUFA*	Quantiity of PUFA*
Lag phase	1.72 ± 0.11[a]	1.07 ± 0.16 [b]	0.57 ± 0.03[a]	0.18 ± 0.03 [b]
Late lag phase	2.12 ± 0.18 [a]	1.23 ± 0.13[b]	0.94 ± 0.07 [a]	0.22 ± 0.05[b]
Log phase	2.78 ± 0.19 [a]	1.47 ± 0.15[c]	1.03 ± 0.08 [a]	0.74 ± 0.06[c]
Late log phase	3.12 ± 0.25 [a]	1.83 ± 0.14[d]	1.62 ± 0.12 [a]	0.96 ± 0.05[d]
Stationary phase	3.08 ± 0.23 [a]	1.68 ± 0.12[e]	1.60 ± 0.11 [a]	0.82 ± 0.02[e]
Late stationary phase	2.17 ± 0.15 [a]	1.50 ± 0.13[c]	1.27 ± 0.09 [a]	1.84 ± 0.07[f]

*% of dry weight [Total of four PUFA – C16:3; C18:4; C20:5; C22:6)]

Each value is the mean ±SD of three replicates. Within each column means with the different superscript letters in all groups are statistically significant (t-test P<0.05 and subsequent posthoc multiple comparison with the SNK test

The statistical analysis shows that the influence of *Psedomonas sp.* inoculation in both the microalgae significantly (P<0.05) increased the PUFA level in microalgae harvested in all the phases when compare with the control

Associated Bacteria and Total PUFA

In this experiment, percentage of individual fatty acid and total PUFA of *Skeletonema* and *Isochrysis* were studied to understand the role of associated bacteria on PUFA content in the algal cells. Four poly unsaturated fatty acids like C16: 3, C18:4, C20:5 and C22:6 were quantified individually from both the micro algae. Among the four fatty acids

quantified in *Skeletonema* C20:5 was the major fatty acid (1.109/100g) inoculated with *Pseudomonas* sp. and in control also the same PUFA was found in maximum quantity (0.89 g/100g) but less than the experimental algae.

When the total PUFA from both experimental and control were quantified, the experimental algae had 3.06 g/100g and in control it was 2.41 g/100g which showed the effect of associated bacteria in increasing the PUFA level in micro algae. Even though the PUFA level normally varied with species of algae, the increase of PUFA due to the action of associated bacteria was evidenced in all the two experimental algae than the control. (Table -6)

Table 6: A Comparison Study of Percentage of Individual Fatty Acid Dry Weight and Total PUFA of Associated Bacteria Inoculated and Non-Inoculated *Skeletonema* sp. and *Isochrysis* sp.

PUFA	*Skeletonema* sp.		*Isochrysis* sp.	
	Pseudomonas Inoculated	Control	*Pseudomonas* Inoculated	Control
C16 : 3	0.48 ± 0.03^{a}	0.37 ± 0.02^{b}	0.32 ± 0.03^{a}	0.24 ± 0.02^{b}
C18 : 4	0.62 ± 0.05^{a}	0.48 ± 0.04^{b}	0.50 ± 0.04^{a}	0.39 ± 0.03^{b}
C20 : 5	1.10 ± 0.09^{a}	0.89 ± 0.08^{b}	0.62 ± 0.06^{a}	0.47 ± 0.05^{b}
C22 : 6	0.86 ± 0.08^{a}	0.67 ± 0.06^{b}	0.58 ± 0.05^{a}	0.35 ± 0.03^{b}
Total	**3.06**	**2.41**	**2.02**	**1.45**

Percentage of dry weight [PUFA is the add up total of four fatty acids] C16:3 – hexadecatrieonic acid methyl ester; C18:4- Octadecatetraenoic acid methyl ester; C20:5 – Eicosapentaenoic acid methyl ester; C22:6 – Docosa hexaenoic acid methyl ester

Each value is the mean ± SD of three replicates within each column means with different superscript letters in both group are statistically significant (t–test<0.05 and subsequent posthoc multiple comparison with SNK test)

The statistical analysis shows that the influence of Psedomonas inoculation in both the microalgae significantly ($P<0.05$) increased the total PUFA on weight basis when compared with the control

DISCUSSIONS

This study was undertaken to know the role of associated bacteria in influencing the physico-chemical nature of the algal oil. This study was conducted because the physico-chemical nature of algal oil varied even provided all physical, chemical and nutritional conditions constant during our study. This simple clue made us to search another biological factor co-existed with the micro algae. To find out the exact bacterial species which encourage the growth of micro algae, the bacterial species co-existed (associated) with the algal culture were isolated and reintroduced into the freshly prepared algal culture devoid of bacteria. The associated bacteria that encouraged the growth of the culture have already been documented by different workers. The bacteria present in microalgae induced the growth of some species of microalgae (Martin.et al., 2005) [21].

Martin *et al*., 2005[21] also studied the relationship between microalgae and associated bacteria. This study stated that as most of the microalgae are the Cobalamin auxotrophs. This Cobalamin is needed to the algae for metabolism and involves in the growth of algae. The source of Cobalamin is only from the associated bacteria and moreover the associated bacteria compete with microalgae for available nutrients such as N and P and at the same time produce vitamin B12 for the algae that are not able to synthesis themselves (Croft, M.,T. et al.,2005). The physico-chemical characters of the algal oil such as solidification point, melting point, specific gravity, moisture content, acid value, ß caroteine and total carotenoids were studied to know the influence of bacteria associated with them. Among several physicochemical characters studied, the associated bacteria influencing only on total oil yield and carotenoid production. The other factor varied based on the species of micro algae; two micro algae studied here showed variation in all the studied parameters. Work conducted by

(Croft *et al*., 2005) also confirmed the same finding. In their study, it has been stated that some of the chemical constituent in the algal cell are based on the type of associated bacteria co-existed in the culture and also the association ship varies based on the extracellular compound secreted by the algae.

The role of associated bacteria on the level of fat soluble vitamin has also been studied and found that the associated bacteria (*Pseudomonas* sp.) enhanced the level of Vitamin A and E and no changes was occurred in Vitamin D. The associated bacteria which enhanced the production of β-carotein in the algal cells have also been documented in this study. As the β- carotein is the precursor of Vitamin A, it is possible for the increase of Vitamin A.

In order to know the influence of associated bacteria on accumulation of minerals like calcium, sodium, potassium, cobalt, iron, magnesium, zinc, sulphur and phosphorus in the oil, the crude oil obtained from the experimental and control algae were tested for the above said minerals and the results proved that there was no influence found in the mineral content of algal oil based on associated bacteria. The quantity of PUFA obtained from the micro algae not only related with the phases of growth of algal culture at which the cells were harvested and species of algae but also the associated bacteria co-existed with the culture place a role on PUFA level in the algal oil. In this experiment, algal oil obtained from the algal cells which was harvested at late log phase possessed the maximum PUFA than the algae harvested at other phases of growth and at the same time, the associated bacteria inoculated algae yielded maximum oil in the algal cells harvested in all the phases of growth when compared with the control.

Among the PUFA quantified in this study, C20:5 (Ecosapentaenoic acid methyl ester) was found to be maximum and followed by C22:6, 48:4, C16:3. All the four quantified fatty acids were found high in experimental algal oil extracted from the algae that was grown co-existed with associated bacteria than the control. This showed the positive role of associated bacteria on the PUFA production in microalgae. The quality of fatty acid in the tested oil content increased due to the presence of selected bacteria; the reason may be the easy availability of some of the chemicals. According to (Croft *et al*., 2005) the variation in the fatty acid composition is mainly due to the availability of some of the trace elements in the external medium more bio-available for algae. Keshtacher-Liebson *et al*.,in 1995 also stated that the bacteria present in the algal culture make some of the nutrients and trace elements bio-available so that the elements can be diffused in to the algal cells and act as a part of enzyme required for the biosynthesis of fatty acids.

CONCLUSIONS

From this study, it has been concluded that some of the particular bacteria living along with microalgae not only increases the growth of algae but also increased thePUFA in the total fatty acid content. Presence of more PUFA in the microalgae makes it more suitable to use as an obligatory live feed in larviculture of fin and shell fishes. The PUFA rich algae not only increases the larval survival but also help to produce healthy larvae which give best performance in grow out system and hence this study opens a new avenue in the larviculture system where this bacteria will definitely play an important role in the production of PUFA rich microalgae.

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